Disintegrin-like domain of glycoprotein B regulates Kaposi’s sarcoma-associated herpesvirus infection of cells

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Kaposi’s sarcoma-associated herpesvirus (KSHV) glycoprotein B (gB) is a lytic structural protein expressed on the envelope of mature virions and on the membrane of cells supporting lytic infection. In addition to this viral glycoprotein’s interaction with integrins via its RGD (Arg-Gly-Asp) motif, KSHV gB possesses a disintegrin-like domain (DLD), which binds integrins as well. Prior to this study, there has been minimal research involving the less common integrin-binding motif, DLD, of gB as it pertains to herpesvirus infection. By using phage display peptide library screening and molecular biology techniques, the DLD of KSHV gB was shown to interact specifically with non-RGD binding \( \alpha _9 \beta _1 \) integrins. Similarly, monitoring wild-type infection confirmed \( \alpha _9 \beta _1 : \text{DLD} \) interactions to be critical to successful KSHV infection of human foreskin fibroblast (HFF) cells and human dermal microvascular endothelial cells (HMVEC-d) compared with 293 cells. To further demonstrate the importance of the DLD of gB in KSHV infection, two recombinant virus constructs were generated using a bacterial artificial chromosome (BAC) system harbouring the KSHV genome (BAC36): BAC36\( _{D} \)-KSHV (lacking a functionally intact DLD of gB and containing an introduced tetracycline cassette) and BAC36\( _{T} \)-KSHV (containing an intact DLD sequence and an introduced tetracycline cassette). Accordingly, BAC36\( _{D} \)-KSHV presented significantly lower infection rates in HFF and HMVEC-d cells compared with the comparable infection rates achieved by wild-type BAC36-KSHV and BAC36\( _{T} \)-KSHV. Thus, the present report has delineated a critical role for the DLD of gB in KSHV infection, which may lead to a broader knowledge regarding the sophisticated mechanisms utilized by virus-encoded structural proteins in KSHV entry and infection.

**INTRODUCTION**

Since the 1994 discovery of Kaposi’s sarcoma-associated herpesvirus (KSHV) in the Chang–Moore laboratory (Chang et al., 1994) 20 years ago, efforts to understand the intricacies of this double-stranded DNA virus have continued. KSHV, also referred to as human herpesvirus-8 (HHV-8), belongs to the gamma-2-herpesvirus subfamily and is the eighth and latest addition to Herpesviridae (Russo et al., 1996). KSHV causes a variety of cancers such as Kaposi’s sarcoma (KS), primary effusion lymphoma and multicentric Castleman disease (Hamden et al., 2005).

In general, envelope-associated glycoproteins predominantly assist virus in the entry process (Bryan et al., 2005). In this study, our focus is on KSHV glycoprotein B (gB), a lytic structural protein primarily expressed on the envelope of mature virions, but also present on the membrane of cells supporting lytic infection (Akula et al., 2001a). KSHV virus binding and entry has been linked to gB mediated interactions not only with cell surface heparan sulfate (HS) molecules but also to integrins, transmembrane receptor molecules with involvement in processes such as adhesion, motility and endocytosis (Akula et al., 2001a, 2002; Hahn et al., 2009). With KSHV being the first herpesvirus shown to exhibit an interaction with adherent target cell integrins—a preliminary step essential for successful viral infection—it is now known that via its RGD (Arg-Gly-Asp) motif, KSHV gB functionally interacts with a variety of cellular integrins, namely \( \alpha _3 \beta _1 , \alpha _v \beta _3 \) and \( \alpha _v \beta _5 \) (Chakraborty et al., 2012). Unlike the RGD of gB, the disintegrin-like domain (DLD) is a less common integrin recognition motif that was initially identified within the human cytomegalovirus (HCMV) envelope gB (Feire et al., 2010). The DLD in gB was found to bear a striking resemblance to the ADAM (a disintegrin and metalloprotease) disintegrin loop (Feire et al., 2004). Members of the ADAM family are multifunctional proteins that contain a metalloprotease domain and a disintegrin motif that confers RGD-independent...
integrin-binding (Feire et al., 2004). It has been observed that KSHV gB possesses a DLD (RX5-7D/ELXXFXSC; 66–85 aa; with a conservative D to E substitution) that is notably conserved among gB homologues of many herpesviruses; specifically beta and gamma herpesviruses (Feire et al., 2010).

Thus, in seeking to delineate a role for the DLD of KSHV gB, we hypothesized the DLD in KSHV gB to play a critical role in the virus infection of cells. Results from our study, using phage display peptide library screening and molecular biology techniques, implicate the ability of the DLD of gB to interact specifically with α9β1 integrins. Utilizing a bacterial artificial chromosome (BAC) system harbouring the KSHV genome (BAC36), we generated two recombinant virus constructs, BAC36_D-KSHV (containing alanine point mutations within the DLD sequence of gB and an introduced tetracycline cassette from vector pEX18TC) and BAC36.T-KSHV (containing an intact DLD sequence and the introduced tetracycline cassette from vector pEX18TC) as a means to decipher a potential role for the DLD of KSHV gB in infection of cells.

RESULTS

Expression and purification of gBΔTMΔD

The KSHV-encoded 2106 bp region of the orf8 gene encoding gBΔTM lacking the transmembrane (TM) and carboxyl domains (Wang et al., 2003) was used to generate a soluble gB lacking a functionally intact DLD, gBΔTMΔD (Fig. 1). This was a crucial step to characterize a role for the DLD of KSHV gB. Coomassie staining of SDS-PAGE gels was conducted to analyse protein purity, and for detection following standard Western blotting protocols (Fig. 2). When purified gBΔTMΔD protein treated with 2-mercaptoethanol (2ME; reducing conditions) was analysed via Coomassie staining, bands of approximately 35–40, 68 and 104 kDa were observed in the lane with gB ΔTM (Fig. 2). Impurities such as other contaminating proteins were not detected in either the gBΔTMΔD or the gB ΔTM preparations. When gBΔTMΔD and gB ΔTM were resolved under non-reducing conditions (~2ME), the 35–40 and 68 kDa bands disappeared while only 104 kDa band and the multiple polypeptides of more than 180 kDa were observed.

![Diagram of gB (orf8) and gBΔTMΔD](http://vir.sgmjournals.org)

**Fig. 1.** Generating KSHV gBΔTMΔD. The diagram shows the schematic of gBΔTM and gBΔTMΔD mutant compared with the full-length KSHV gB (orf8).
The migration pattern of gB_DTM_D was comparable to what was observed when gB_DTM was resolved in earlier studies (Dyson et al., 2010; Wang et al., 2003). The specificity of the gB proteins was confirmed by performing Western blotting experiments (Fig. 2). This implies that gB_DTM_D, like gB_DTM, expressed in Spodoptera frugiperda (Sf9) ovarian cells can form disulfide-linked dimers or multimers under non-reducing conditions. These results suggest that the alanine point mutations introduced in the DLD sequence of gB did not significantly change the molecular mass or the migration pattern of the protein on gels compared with the wild-type (gB_DTM).

**Phage display (PhD) peptide library identifies integrin α9 as a potential receptor for DLD in gB**

The PhD phage display library was used to identify novel ligands for the DLD in gB. Three random peptide libraries, a linear (X)7, a cyclic Cys (X)7 Cys and a linear (X)12 were screened against the gB DLD peptide. The single most common peptide (based on the increased frequency; 20 out of 30) possessed a PKA(P)DGR(H)V(L) sequence (Table 1). Interestingly, the major conserved sequence among the peptides identified had a conserved sequence of PKADGRV (9 out of 30).

We tested the ability of the phage carrying peptides L3 (PKADGRV), F1 (DCKPKPDGRLRD) and F5 (PKADGHV) to bind gB_TDM immobilized on 96-well plates. It was determined that the phage carrying peptides L3, F1 and F5 bound gB_TDM more efficiently than BSA and gB_DTM_D (Fig. 3a). However, the phage encoding peptide PKADGRV (peptide L3) bound more efficiently than the other peptides (F1 and F5). The binding of the phage encoding L3 peptide to gB_TDM could be significantly blocked by including 1 mM of synthetic peptide PKADGRV during the incubation step compared with using scrambled peptide (Fig. 3b). The effect of PKADGRV peptide was measured against the known positive control, gBDLD peptide (Fig. 3b). A BLAST protein search of this sequence identified a integrin (Homo sapiens; NCBI Ref Seq: NM_002207.2) to possess such a motif (133–139 aa). This was an interesting finding, because at the beginning of the study we had predicted a non-RGD binding integrin (Yokosaki et al., 1994) as a probable receptor capable of binding the DLD of gB.

**Table 1. Amino acid sequences of the phage-displayed peptides isolated by screening against DLD in gB**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Frequency</th>
</tr>
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<tbody>
<tr>
<td>L3</td>
<td>PKADGRV</td>
<td>9</td>
</tr>
<tr>
<td>L5</td>
<td>MTAENIR</td>
<td>1</td>
</tr>
<tr>
<td>F1</td>
<td>DCKPKPDGRLRD</td>
<td>6</td>
</tr>
<tr>
<td>F3</td>
<td>QAMSDKFRCGWA</td>
<td>2</td>
</tr>
<tr>
<td>F5</td>
<td>PKADGHV</td>
<td>5</td>
</tr>
<tr>
<td>F9</td>
<td>CNHPLEC</td>
<td>1</td>
</tr>
<tr>
<td>S6</td>
<td>PYHDQIA</td>
<td>1</td>
</tr>
<tr>
<td>S8</td>
<td>LRPADGPTEFW</td>
<td>2</td>
</tr>
<tr>
<td>S9</td>
<td>SWADTTIQYVVL</td>
<td>1</td>
</tr>
<tr>
<td>S4</td>
<td>RFIYPEDPFIEC</td>
<td>1</td>
</tr>
</tbody>
</table>
Fig. 3. Phage encoding peptide PKADGRV bound gB\(\Delta\)TM efficiently. (a) p.f.u. recovered when 10\(^{-11}\) p.f.u. of phage carrying peptides L3, F1 and F5 were screened against immobilized g\(\beta\)\(\Delta\)TM, g\(\beta\)\(\Delta\)TM\(\Delta\)D, or BSA (negative control) proteins on microplates. (b) p.f.u. recovered when 10\(^{-11}\) p.f.u. of phage carrying peptide L3 was screened against immobilized g\(\beta\)\(\Delta\)TM, g\(\beta\)\(\Delta\)TM\(\Delta\)D, or BSA on microplates in the absence or presence of 1 mM solution of PKADGRV, gBDLD peptide, or scrambled peptide. Finally, ELISA was performed to determine the interactions between (c) \(\alpha 9/1\) or \(\alpha V/3\) (d) with immobilized g\(\beta\)\(\Delta\)TM, g\(\beta\)\(\Delta\)TM\(\Delta\)D, BSA, or GST. Each point denotes the average ±SD (error bars) of three experiments. Columns with different alphabets and asterisks on the data points denote the value to be statistically significant (\(p<0.05\)) by least significant difference (LSD). (e) Immunoprecipitation experiments to demonstrate gB interactions with \(\alpha 9/1\). Recombinant g\(\beta\)\(\Delta\)TM (lane 1) compared with g\(\beta\)\(\Delta\)TM\(\Delta\)D (lane 3) specifically bound \(\alpha 9\). As a positive control, gB interactions with \(\alpha V/3\) was tested.
Plate-based binding assays demonstrate the DLD of KSHV gB to bind α9β1

The integrin α9 commonly forms a heterodimer with β1 integrin subunit, α9β1 (Young et al., 2001). Upon identifying the α9 integrin as a plausible receptor for gB, we used various modified ELISAs to test the ability of soluble KSHV gB to bind α9β1. The binding of α9β1 to gBαTM was monitored using polyclonal antibodies to α9β1 (H-198). ELISA studies identified α9β1 to bind specifically 1 µg ml⁻¹ of gBαTM in a dose-dependent manner (Fig. 3c) compared with gBαTMΔD and non-specific controls, BSA or GST. Similar data were observed when monoclonal antibodies to α9β1 (clone #560201) were used in the ELISA (data not shown). Interestingly, ELISA studies demonstrated an RGD binding integrin αvβ3, to bind gBαTM and gBαTMΔD to comparable extent (Fig. 3d), demonstrating a functional RGD motif in both the soluble forms of the gB tested. Based on these results, 1 µg ml⁻¹ of both gBαTM and α9β1 were used in all our other experiments described below. An immunoprecipitation experiment was done to authenticate further the results from ELISA-based assays. Herein, the α9 subunit was found to bind specifically gBαTM (Fig. 3e, lane 1) and not the gBαTM lacking a functional DLD (Fig. 3e, lane 3).

To confirm the specificity of the KSHV-encoded gB:α9β1 binding, we attempted to neutralize this interaction by conducting competitive ELISAs. In this case, different concentrations of various ligands or antibodies known to interact with gB, the DLD motif specifically, or α9β1 were used. KSHV gB is known to interact with HS via a charge based interaction (Bryan et al., 2005). HS, chondroitin sulfate-A or -B (CSA, CSB; control glycosaminoglycans) had little effect on the gB:α9β1 interactions, as binding between gBαTM and α9β1 still occurred (Fig. 4a).

KSHV gB interacts with a variety of integrins via its RGD domain. In order to determine if gB interactions via the RGD domain altered its ability to bind α9β1, competitive ELISA using GRGDSP and KQAGDV (an irrelevant peptide) was performed. The results confirmed that increasing concentrations of RGD peptides did not alter the ability of α9β1 to bind gBαTM (Fig. 4b). Interestingly, the RGD peptide significantly blocked the ability of αvβ3 to bind gBαTM (Fig. S1, available in the online Supplementary Material).

VEGF and tenasin C are common known ligands for integrin α9β1 (Andrews et al., 2009; Vlahakis et al., 2005). Our data suggest that increasing concentrations of VEGF and laminin did not alter the binding of α9β1 to gBαTM.

![Fig. 4](image-url)
Inhibiting interactions between α9β1 and the DLD of gB lowers KSHV infection

To confirm a critical role for the DLD:α9β1 interactions in KSHV infection we utilized the recombinant KSHV that expressed green fluorescent protein (GFP) referred to as rKSHV.152 (Akula et al., 2001b; Grange et al., 2012; Vieira et al., 2001) and three different cell types known to support KSHV infection of cells. They were human foreskin fibroblasts (HFFs), human embryonic kidney 293 epithelial cells (293 cells) and human microvascular endothelial (HMVEC-d) cells. First, we determined if these cells actually expressed the inserted tetracycline cassette in BAC36 and BAC36.T clones (Fig. S5A). All of the recombinant viral genomes contained the orf8 gene as determined by PCR (Fig. S5B). Second, we confirmed that targets 1 and 2, representing a portion of orf7, the complete sequence of orf8 and the N-terminal sequence of orf9 from the original BAC36 genome (Fig. S5B), were contained in BAC36ΔD and BAC36.T (Fig. S5C). Third, we amplified orfβ from the BAC36, BAC36ΔD, BAC36.T genomes using T2(R) and T2(F) primers. As predicted, we amplified a 4005 bp DNA fragment in the BAC36 genome, while amplifying a product of size 5698 bp from both BAC36ΔD and BAC36.T (Fig. S5D). The above results were authenticated by sequencing using appropriate primers to confirm the specific mutations in the orf8 gene contained within the BAC36ΔD (Fig. S5E).

DLD of gB is critical to KSHV infection of cells

We hypothesized that knocking down a functional DLD of gB in KSHV will result in a decrease in virus infection of cells. To test this hypothesis, we developed a recombinant virus that lacked a functional DLD in KSHV gB (BAC36ΔD-KSHV). As a control to the BAC36ΔD-KSHV, we also generated BAC36.T-KSHV that had an uninterrupted and functional gB, but with a tetracycline cassette (as in BAC36ΔD) introduced in the intron region between the orfβ and orf9. In brief, by employing overlap PCR (Fig. 6a, b), a series of cloning experiments and then recombination rendered BAC36ΔD and BAC36.T clones, respectively (Fig. 6c). Prior to transformation via electroporation and tetracycline selection stages, the correct orientation of the inserted tetracycline cassette in orfβDLD,Tet/TOPO and orfβ17.8.A9.Tet/TOPO positive clones was confirmed by restriction enzyme digestion using BamHI and NheI, clone #3,2 and clone #7,2, denoting orfβDLD,Tet/TOPO and orfβ17.8.A9.Tet/TOPO respectively, contain the correctly oriented cassette and were subsequently used in the generation of BAC36ΔD and BAC36.T clones (Fig. S5A). These clones were further compared with the BAC36 wild-type and confirmed by performing a variety of PCRs. First, PCR amplified tetracycline gene in BAC36ΔD and BAC36.T compared with the BAC36 (Fig. S5B). All of the recombinant viral genomes contained the orf8 gene as determined by PCR (Fig. S5B). Second, we confirmed that targets 1 and 2, representing a portion of orf7, the complete sequence of orf8 and the N-terminal sequence of orf9 from the original BAC36 genome (Fig. S5B), were contained in BAC36ΔD and BAC36.T (Fig. S5C). Third, we amplified orfβ from the BAC36, BAC36ΔD, BAC36.T genomes using T1(F) and T2(R) primers. As predicted, we amplified a 4005 bp DNA fragment in the BAC36 genome, while amplifying a product of size 5698 bp from both BAC36ΔD and BAC36.T (Fig. S5D). The above results were authenticated by sequencing using appropriate primers to confirm the specific mutations in the orf8 gene contained within the BAC36ΔD (Fig. S5E).
Fig. 5. Role of integrin α9β1 in wild-type KSHV infection. (a) HFF, HMVEC-d and BCBL-1 cells express α9. cDNA synthesized from target cells was subjected to PCR analysis to amplify a α9 PCR product (α9: ~190 bp) that was resolved in a 2.1% agarose gel. (b) Flow cytometry analysis of the surface expression of α9 integrin subunit in 293, HFF and HMVEC-d cells was performed following staining with Integrin α9 (H-198) rabbit polyclonal antibody and subsequent incubation with goat anti-rabbit FITC. The average percentage of cells positive for the α9 expression from three independent experiments is shown above the marker. Representative histogram plots show interactions with pre-immune IgG (purple plot) and anti-α9, (green outline) for each cell type. (c–e) Inhibition of rKSHV.152 infection by 20 µg ml⁻¹ of antibodies to integrins (c), soluble integrin (d) and 30 µg ml⁻¹ of anti-DLD antibodies (e) is shown. In the above experiments (panels c–e), infection was monitored at 72 h p.i. by recording the total number of cells expressing GFP under a fluorescent microscope. Data are presented as percentage of inhibition of the virus infectivity obtained when the cells were pre-incubated with Dulbecco’s modified eagle medium as a control. Data are the means ± SD (error bars) of three experiments. Letters above columns indicate statistical significance (P<0.05) by LSD.
We then tested the infection rates of the above different recombinant viruses generated in the lab. First, we analysed cell surface expression of gB in BAC36-KSHV and BAC36ΔD-KSHV infected cells by FACS. This is vital, as the results demonstrated that cell membrane expression of gB in 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced 293 cells infected with BAC36-KSHV and BAC36ΔD-KSHV was comparable (Fig. 7a). Next, we monitored infection of BAC36-KSHV, BAC36ΔD-KSHV, BAC36.T-KSHV in 293, HFF and HMVEC-d cells as per standard procedures. Our results indicated a sharp decline in the BAC36ΔD-KSHV infection of HFF and HMVEC-d cells compared with BAC36-KSHV and BAC36.T-KSHV, respectively (Fig. 7b). Interestingly, BAC36ΔD-KSHV infection of 293 cells was not altered compared with BAC36-KSHV and BAC36.T-KSHV (Fig. 7b). The numbers of GFP positive cells at 72 h.p.i. of BAC36-KSHV in 293, HFF and HMVEC-d cells were 116, 28 and 57, respectively. Taken together, our results implicate a critical role for DLD of gB in KSHV infection of HFF and HMVEC-d cells.

**DISCUSSION**

In addition to the most common integrin recognition motif, RGD (Akula et al., 2002; Garrigues et al., 2008), KSHV gB also possesses DLD juxtaposed in the extracellular amino terminal coil region that also has potential integrin-binding capabilities (Feire et al., 2004). In fact, it was the analysis of the related domains of snake venom metallopeptases (SVMPs) that sparked the assumption that other varieties of DLDs, such as in ADAMs, would also be involved in integrin-mediated interactions (Lu et al., 2010; Wolfsberg et al., 1995).

The DLD of KSHV gB (66–85 aa) corresponds to 49–68 aa residues within the EBV gB. EBV is a human herpesvirus closely associated to KSHV, and both are classified as gamma herpesviruses. Earlier, the ectodomain (23–685 aa out of a full 1–875 aa length) of EBV gB was crystallized (Backovic et al., 2009). It was determined that the major portion (52–68 aa) of the electron-dense DLD of EBV gB is contained within the domain III region, which is exposed and actually wraps around the helices to form a left-handed twist. Based on these findings, we predict the DLD of KSHV gB to also be an exposed ectodomain available for interactions with host cell receptor molecules. Outside HCMV, the role of DLD in virus entry has been minimally explored. Thus, this study has sought to unearth the role of DLD of gB in KSHV infection.

Instead of employing antibody-based assays, we utilized phage display peptide library screening to ascertain the putative receptor for the DLD of KSHV gB. We determined the DLD of gB to interact with the host cell receptor molecule, integrin α9, by panning random libraries of phage displayed peptides against the gBDLD peptide fragment (Table 1; Fig. 3a, b). The results from screening the phage display peptide libraries were further authenticated by performing plate-based binding assays (ELISAs) and immunoprecipitation experiments using both gBΔTM and gBΔTMΔD (Fig. 3c, d).

The subunit α9 has been widely shown to combine with β1 to form a single heterodimer (Young et al., 2001) with non-RGD binding capabilities (Yokosaki et al., 1994). For this study, insight regarding specificity of KSHV gB:α9β1 interactions was provided from results of several competitive ELISAs. HS (Fig. 4a), a target cell membrane molecule whose interaction with KSHV is mediated in part by envelope gB (Bryan et al., 2005), VEGF, a known ligand for α9β1 (Vlahakis et al., 2005), or laminin, an extracellular matrix protein (Fig. 4c), did not block the gBα9β1 interactions. Another known ligand for α9β1, tenasin C (Andrews et al., 2009), also failed to neutralize the gBα9β1 interaction (Fig. 4c). In the case of tenasin C, however, enhanced binding was observed between gBΔTM and α9β1 in what we believe to be a result of an allosteric interaction (Fig. 4c), as suggested by an earlier report (Laskowski et al., 2009). Tenasin C is an extracellular matrix (ECM) molecule that is frequently expressed at elevated levels in solid tumours and is said to have a role in cancer formation (Orend & Chiquet-Ehrismann, 2006). Further studies will focus on appreciating gB:α9 interactions with respect to tenasin C expression.

Likewise, competitive ELISAs also confirmed the ability of KSHV gB to interact with integrin α9β1 independent of its RGD domain (Fig. 4b). Our findings are reminiscent of results produced by Eto et al. (2002) who found that mutating the RGD motif of the aforementioned ADAM-15, had no effect on the binding of α9β1 to the protein’s disintegrin domain. Moreover, when using an antibody directed against the DLD in gB, competitive ELISA data showed a substantial downregulation of the gBα9β1 interactions, suggesting the specificity of this antibody (Fig. 4d). These results imply that the avid binding between gB and α9β1 is in fact dependent on the DLD of KSHV gB (Fig. 4d).

In an effort to extrapolate plate-based assays to viral infection, we attempted to test the role of integrin α9β1 in wild-type KSHV infection of different cells (Fig. 5). HFF and HMVEC-d cells express α9β1, but 293 cells do not. KSHV infection of 293 cells was not altered by antibodies to α9, β1 subunits, DLD target sequence, and soluble α9β1 (Fig. 5c, e). In HFF cells, antibodies to α9, β1 subunits, DLD target sequence, and soluble α9β1, significantly lowered KSHV infection (Fig. 5c e). In HMVEC-d cells, there was a significant decrease in KSHV infection of cells due to antibodies against β1 subunit and the DLD target sequence, with only a modest decrease in infection noticed due to antibodies against α9 and soluble α9β1 (Fig. 5c, e). Taken together, from the above results we conclude the following. (i) The α9β1:DLD of gB interactions may be required for an efficient KSHV infection of HFF cells. The DLD interactions may well play a supportive role to the RGD interactions with integrin(s) (Chandran, 2010; Veetil et al., 2008) in promoting virus entry. (ii) There may be another non-RGD...
Fig. 6. Legend on following page.
binding integrin receptor(s) with which DLD of gB interacts in promoting virus infection of HMVEC-d cells. (iii) KSHV utilizes diverse mechanisms to enter a variety of target cells.

The use of soluble integrins and antibodies to define a crucial role for a receptor molecule is not without its limitations, primarily depending upon the purity, function and concentrations of the recombinant proteins or the antibodies. Hence, we generated BAC36ΔD-KSHV (KSHV lacking the functional DLD) to appreciate more completely the physiological role for DLD in virus infection of cells (Fig. 7). Recently, upon sequencing the KSHV-BAC36 genome in its entirety, Yakushko et al. (2011) discerned a 9 kb long unique region (LUR) fragment duplication in the terminal repeat region of several viral stocks acquired by laboratories. However, we assume this did not complicate our generation of BAC36ΔD and BAC36.T, as our modifications to BAC36 did not involve mutagenesis to viral genes located within the potential LUR duplication. Here, the infection of BAC36ΔD-KSHV was compared with BAC36-KSHV wild-type and BAC36.T-KSHV in 293, HFF and HMVEC-d cells (Fig. 7). The results indicated comparable infection rates for BAC36-KSHV and BAC36.T-KSHV in all tested cell types (Fig. 7). However, the infection rates for virus lacking an intact DLD of gB were significantly lower in HFF and HMVEC-d cells compared with 293 cells (Fig. 7), which provides evidence that the DLD of KSHV gB and its interaction with z9β1 has a substantially important role in regulating virus infection. Our results also confirm KSHV to utilize a mechanism of entry into 293 cells that is independent of z9β1. Earlier studies also determined KSHV infection of 293 cells to be via binding heparin sulfate, but independent of RGD integrins (Inoue et al., 2003). At this stage, we can only hypothesize that such an efficient internalization of KSHV by 293 cells occurs as a result of a dynamic and biologically active cell membrane of a transformed cell line compared with primary cells such as HFF and HMVEC-d cells.

Multiple studies have determined that interactions of the gB RGD with z3β1, zVβ3 and zVβ5 are necessary for KSHV entry (Chandran, 2010; Veettil et al., 2008). KSHV has also been shown to use DC-SIGN and the 12-transmembrane glutamate/cysteine exchange transporter protein xCT as receptor molecules in dendritic cells, macrophages and activated B cells (Rappocciolo et al., 2008, 2006; Zhang & Gao, 2012). Recent studies by Hahn et al. (2012) deciphered a key role for gH/gL interactions with EphA2, a tyrosine kinase, in promoting virus entry. Like other viruses, KSHV has evolved to utilize different combinations of host cell entry mechanisms.

**Fig. 6.** Architecture and generation of recombinant BAC36ΔD. (a) Diagram of how the target 3 PCR product was obtained. (b) DNA agarose gel electrophoresis of purified targets to confirm predicted fragment sizes. Elution-purified target 1 (T1; 2967 bp), target 2 (T2; 1055 bp) and gel-purified target 3 (T3; 4005 bp) were resolved in 1% agarose gels and stained by ethidium bromide. Bands of expected sizes were rendered. (c) Schematic depiction of the molecular biology procedures involved in the construction of BAC36ΔD and BAC36.T clones. A detailed description concerning the construction of the clones is provided in the online Supplementary Material.

**Fig. 7.** DLD of gB is critical for KSHV infection. (a) Monolayers of 293 cells were infected with 0.1 m.o.i. of BAC36-KSHV or BAC36ΔD-KSHV. At 48 h.p.i., the cells were treated with TPA for 72 h. These cells were analysed for the surface expression of gB in 293 cells by staining with pre-immune IgG (solid purple) or rabbit polyclonal anti-gB antibody (green outline) followed by incubation with goat anti-rabbit FITC before examining by FACS. The average percentage of cells positive for the surface expression of gB from three independent experiments is shown above the marker. A representative histogram plot for each cell type is depicted. (b) Monolayers of 70–80% confluent 293, HFF and HMVEC-d cells were infected with BAC36-KSHV, BAC36ΔD-KSHV, or BAC36.T-KSHV. At 72 h.p.i., the total number of cells expressing GFP was counted under a fluorescent microscope. The percentage of target cells infected with BAC36-KSHV is shown. Data shown are the means ± SD (error bars) of three experiments. Letters above columns indicate statistical significance (P<0.05) by LSD.
receptor molecules to infect target cells, and integrin α9β1 could well be the latest addition to KSHV’s arsenal of host cell receptor molecules utilized for entry.

Though these findings delineate a critical role for the lesser studied integrin-binding domain (DLD) of gB in KSHV infection, this study has also led to the opening up of other questions that await our further research. Importantly, we seek clarity regarding the manner by which the α9β1:DLD-induced cellular signalling alters initial stages of virus infection (i.e. virus binding, initial target cell entry, escape to the endosome, or eventual nuclear transport). Additionally, we seek to understand how reactions involving DLD of gB and integrins support RGD-dependent interactions critical to virus entry. Do these seemingly mutually exclusive integrin-binding motifs within gB somehow work in concert to regulate virus infection? Moreover, ongoing studies will also monitor the possibility of integrin heterodimer α9β7 interaction with DLD of KSHV to regulate virus infection, much like the ability of ADAM-2 in RPMI 8866 cells (which express little or no β1) to interact with α9β7 (Desiderio et al., 2010). All further studies in this area will strive for a better understanding of the intricacies involved in the role of, and mechanisms utilized by, glycoproteins in KSHV entry and infection.

**METHODS**

**Cells.** HFF cells, 293 cells, HMVEC-ds (CC-2543; Clonetics) and Sf9 ovarian cells were propagated as per standard laboratory protocols (Akula et al., 2005).

**Antibodies.** An antibody to DLD peptide sequence of gB (anti-DLD) was generated in rabbits by Pi-Proteomics and used in ELISAs performed in this study. Rabbit antibodies to the RGD-containing sequence of gB (anti-RGDgB-N1) and a peptide sequence from the C-terminal domain in gB (anti-gB-C) were also used. Antibodies to full-length gB, RGDgB-N1 and gB-C have been described in earlier studies (Akula et al., 2002). Human α9 (H-198) rabbit polyclonal antibodies (Santa Cruz Biotechnology), α9 monoclonal mouse IgG clone 960201 (R & D Systems), αv monoclonal mouse IgG (clone P3G8; Millipore), α5 monoclonal mouse IgG clone (clone P1D6; Millipore) and β1 monoclonal mouse IgG (clone 656; Millipore), were also used in this study.

**Proteins and peptides.** See the online Supplementary Material for a full description.

**Cloning and expression of recombinant gBΔTM\DLD.** His-tagged, recombinant and soluble KSHV gBΔTM and gBΔTM lacking the DLD (gBΔTMAD) were expressed and purified from Sf9 cells as per earlier studies (Dyson et al., 2010; Wang et al., 2003).

**Western blotting.** Equal concentrations of soluble gB proteins (25 μg) were resolved on SDS-PAGE gels prior to being transferred to a PVDF membrane that was probed using rabbit polyclonal antibodies to gB and appropriate secondary antibodies as per earlier studies (Dyson et al., 2012).

**PCR.** PCR assays were conducted using synthesized cDNA and specific primers (Table 2). PCR amplifications were performed using Platinum Taq DNA Polymerase, High Fidelity (Life Technologies) and/or Advantage cDNA PCR kit (BD Biosciences Clontech) at appropriate annealing temperatures and extension times. Amplified products were separated on agarose gels, and expression was monitored.

**Screening phage display peptide libraries to determine a novel receptor for gB.** A detailed protocol is provided in the online Supplementary Material.

**ELISA.** To characterize the binding interactions between soluble gB and integrin α9, ELISA was performed as per standard protocols. A detailed protocol is provided in the online Supplementary Material.

**Immunoprecipitation.** Soluble integrins (1 μg ml⁻¹) were incubated with different forms of 1 μg ml⁻¹ of recombinant gB for 2 h at 4 °C.

**Table 2. List of primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>x9.FW2,Q.F</td>
<td>GATGAGTGGATGGGGGTGAG</td>
</tr>
<tr>
<td>x9.FWQ,R</td>
<td>CCGTGTTCTCCTCCCTAGCT</td>
</tr>
<tr>
<td>DLD-M2 (F)</td>
<td>TCGATCCCGGGAGAGCCGCGCGCGCGAGACCTGAGACAG</td>
</tr>
<tr>
<td>DLD-M2 (R)</td>
<td>CGTCTGCTCCAGGTTCGGCGCGCGCGCTCCCCGGTGATCGA</td>
</tr>
<tr>
<td>pHHV8gB (F)</td>
<td>ACGTGGATCCCCAACATGACCTCCAGG</td>
</tr>
<tr>
<td>ORF8.HIS (R)</td>
<td>TCCGATTTCTCAATAGTAGTATAGTATAGTGCCACCAGGTGTCCTC</td>
</tr>
<tr>
<td>ORF8.RD (F)</td>
<td>ATGACTCCCCAGGTCTAGAAT</td>
</tr>
<tr>
<td>ORF8.D (F)</td>
<td>AAGCACTCTGCTCTCAAGATGT</td>
</tr>
<tr>
<td>ORF8.RD (R)</td>
<td>TCGTGGGCAAAGTGGAAA</td>
</tr>
<tr>
<td>ORF50P8.ChIP.OD (F)</td>
<td>ATCAGGGGACTCTTAAAGC</td>
</tr>
<tr>
<td>ORF50P8.ChIP.OD (R)</td>
<td>GTGCGTCTGTCGACAGTATT</td>
</tr>
<tr>
<td>T1 (F)</td>
<td>GTGACGCTGGTGCTTGCTTGCTTGCTTGCTTGCTTGCGCGGCTGCTTT</td>
</tr>
<tr>
<td>T1 (R)</td>
<td>TCGAATATGATATAGTACCTCCCGGGTTCCGGGACTGATGTC</td>
</tr>
<tr>
<td>T2 (F)</td>
<td>GAGTGTGACTGATGATGAGGTAGTTATGTTGATGTAATT</td>
</tr>
<tr>
<td>T2 (R)</td>
<td>CGGTTGGAAGAACCTCTGCGCCTACAATCTACTAATC</td>
</tr>
<tr>
<td>Tet (F)</td>
<td>CATATGCGCGATTATGGTGCACTCTAGTAC</td>
</tr>
<tr>
<td>Tet (R)</td>
<td>CATATGCGGGATATCGGTCGAGCTCGG</td>
</tr>
</tbody>
</table>
The protein complexes were immunoprecipitated with appropriate antibodies (anti-gB, anti-gV, or pre-immune IgG) for 1 h at 4 °C, followed by addition of 100 μl of swollen Protein A-Sepharose beads and further incubation for another hour at 4 °C. The beads were washed four times with Gold lysis buffer, boiled in sample-loading buffer and resolved by 10% SDS-PAGE gels. Proteins were transferred onto a PVDF membrane and Western blotted using appropriate antibodies as per previously described protocols (Akula et al., 2002). The molecular masses of gV and g9 protein bands are 128 and 140 kDa, respectively.

Generating recombinant KSHV. A detailed protocol is provided in the online Supplementary Material.

Monitoring KSHV infection of cells. KSHV infection of different cells was recorded by counting the number of cells expressing GFP that is indicative of rKSHV.152 and BAC36 infection (Akula et al., 2001b, 2004; Grange et al., 2012).

Flow cytometry. Target cells were washed, incubated in growth medium at 37 °C for 30 min, centrifuged and resuspended in cold PBS. The entire procedure involved the use of cold reagents and temperatures of +4 °C. Cells (1 × 10^6) were incubated with different antibodies at 4 °C for 30 min, washed, incubated with FITC-conjugated appropriate secondary IgG at 4 °C for 30 min, washed and analysed in a FACScan flow cytometer (Becton Dickinson) with appropriate gating parameters.

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REFERENCES


